MMTN
MEDICAL MYCOLOGY
TRAINING NETWORK

Non–culture-based identification

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ASIA FUNGAL WORKING GROUP
an ISHAM working group

ISHAM
INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

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Hot topics in Asian medical mycology

NON CULTURE BASED IDENTIFICATION

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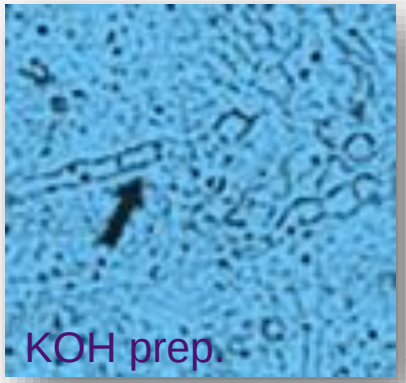
Outline

- Antigen-Antibody detection
  - ELISA
  - Lateral Flow assay
  - Mannan/Galactomannan
  - (1,3)-β-D-glucan
- DNA based: PCR, Real time PCR
- Histopathology: H&E staining, GMS and

Fungal Identification & Diagnosis

- **Standard method** Fungal isolation and cultivation
  - Macro- / Microscopic examination
  - Biochemical test (Yeast)



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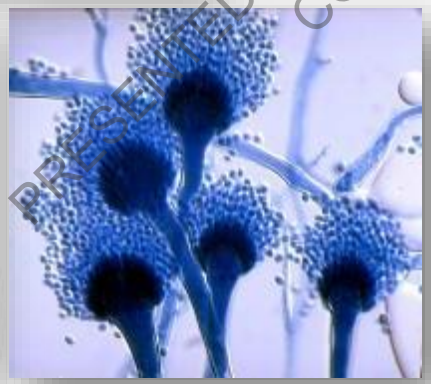
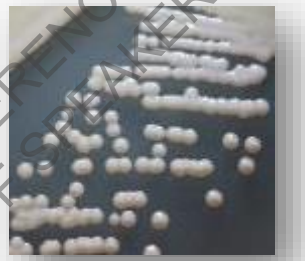
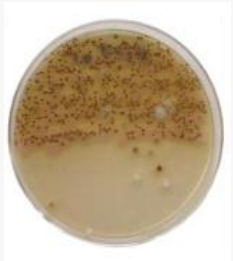


Aspergillus fumigatus



Round cells
Capsule +ve
Urease +ve
Melanin +ve
API: Cryptococcus



Cryptococcus neoformans

Laboratory Diagnosis

APPROACH	PRO	CON	IDENTIFICATION LEVEL
1. CLASSICAL **1.1 Direct examination** - KOH prep., India ink staining - Calcofluor white staining - - Gram stain, MAFB stain	Rapid (1 hr), low cost	Experience	Morphology: Yeast, Mold: non septate, septate: (hyaline/dematiaceous)
1.2 Histopathology - Wright stain - H & E, PAS, Gram, GMS, etc	Tissue reaction Automate	Specialty	Tissue reactions & Morphology
1.3 Culture	Low cost, susceptibility	Time consuming	Genus, species??
2. SEROLOGY 2.1 Antigen (cell wall) - Mannan, GM, BG, 2.2 Antibody : Mannan	One day	False positive, False neg, Ag & Ab together	Group Immunocompetent
3. NON - CLASSICAL **3.1 DNA based** - PCR, FISH, qPCR, sequencing, etc.	- High sens., spec. , Accurate - Both detection & identification	Contamination	Genus, level, typing
3.2 Protein based	Rapid (30 min)	Data based, high cost	Genus, species, developing to detection,

Cell wall composition VS diagnosis tool

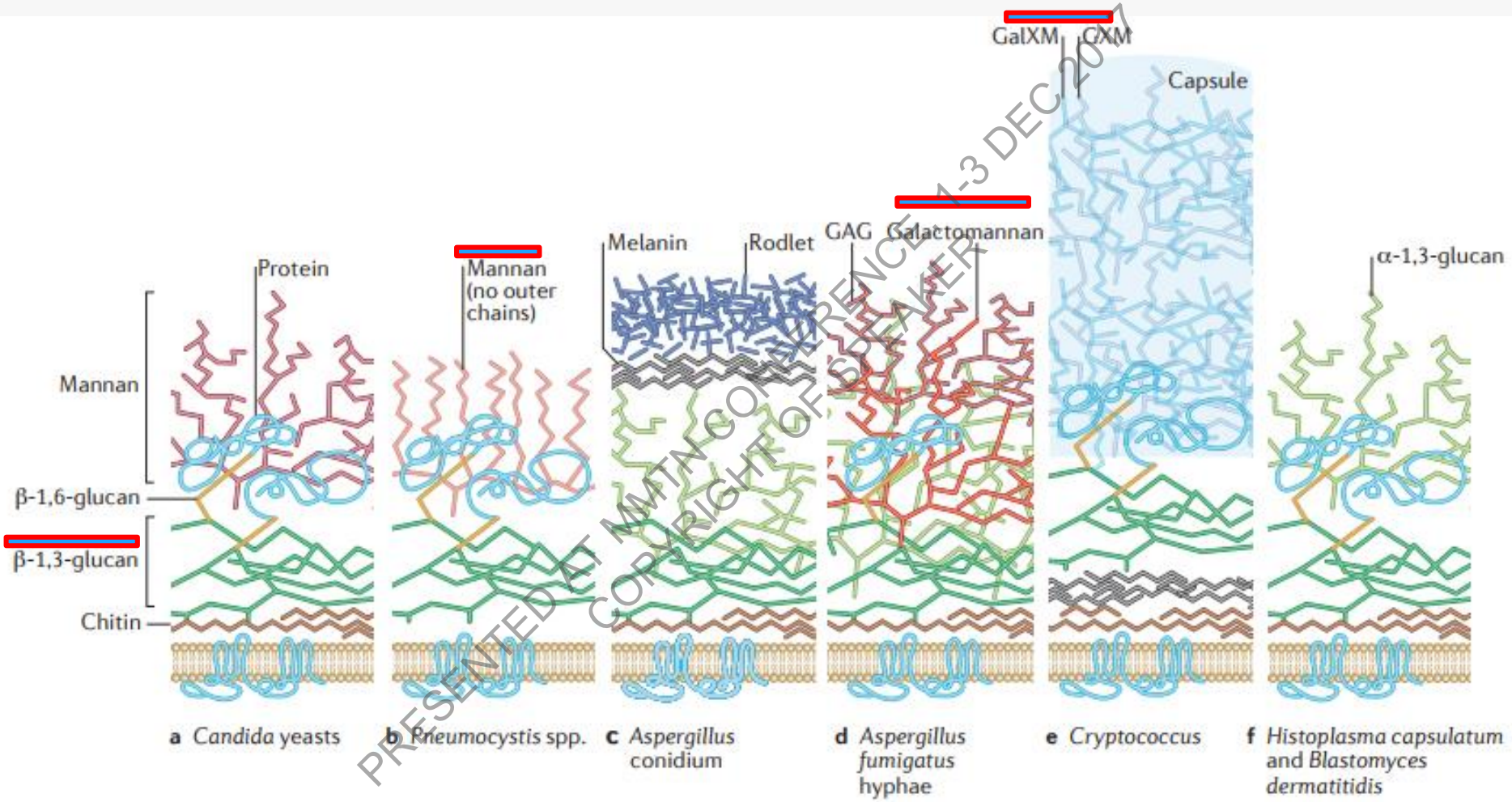


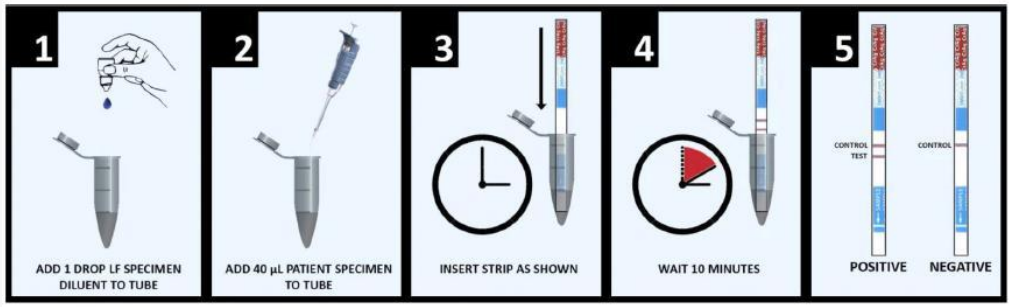
Figure 3 | **Fungal cell wall composition and recognition by phagocytes.** The cell wall structures of selected fungal cells

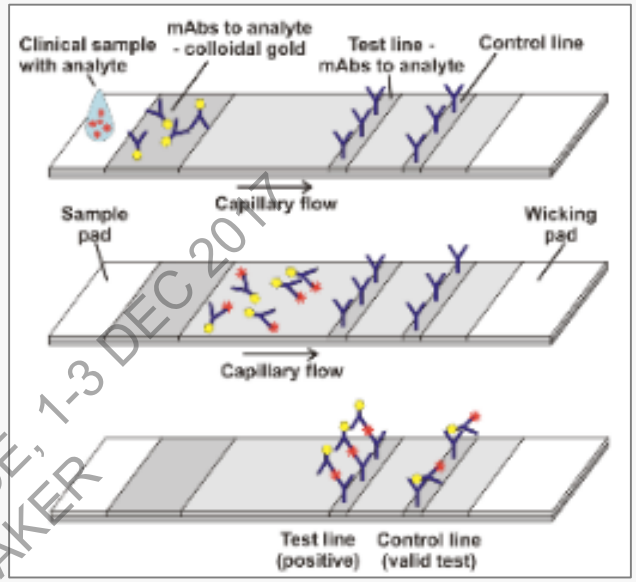
Erwig et al. Nature Reviews Microbiology. 2016. V14: 163-176

Antigen Detection / Antibody detection

- Cryptococcosis
  - GXM (Lateral Flow Strip)
- Histoplasmosis
  - Histoplasma antigen (EIA)
- Invasive Candidiasis
  - Mannan (ELISA) / Anti-Mannan
- Aspergillosis
  - Galactomannan (ELISA)
- Fungal infection
  - (1,3)-β-D-glucan (Kinetic ELISA)

Cryptococcosis: Lateral Flow immunoassay



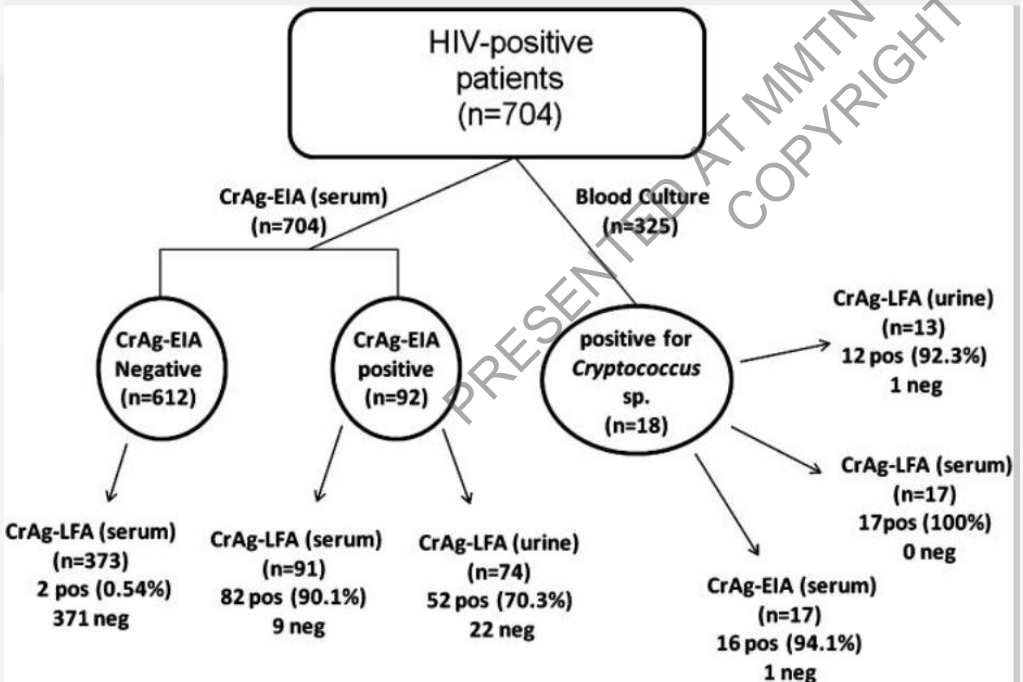


Evaluation of a Newly Developed Lateral Flow Immunoassay for the Diagnosis of Cryptococcosis

Mark D. Lindsley,[1] Nanthawan Mekha,[2] Henry C. Baggett,[3] Yupha Surinthong,[2] Rinrapas Autthateinchai,[2] Pongpun Sawatwong,[3] Julie R. Harris,[1] Benjamin J. Park,[1] Tom Chiller,[1] S. Arunmozhi Balajee,[✉1] and Natteewan Poonwan[2]

Author information ► Article notes ► Copyright and License information ►

Clin Infect Dis. 2011



- Capsular antigen : GXM 4 serotypes
- Diagnosis from CSF, Serum,
- Urine vs Blood culture:
  - 92% sensitivity
- Urine: LTA vs EIA
  - 72% sensitivity
- HIV & Non HIV patients
- Rapid (15 min.), user friendly,
- Point of care assay
- Qualitative, Semi-quantitative assay

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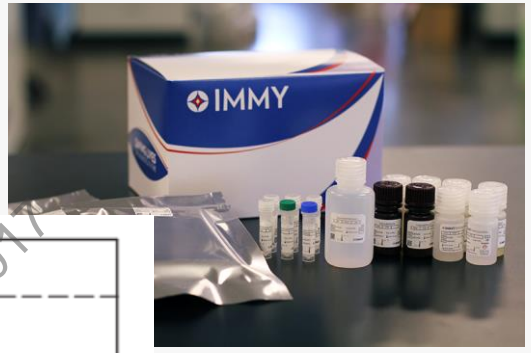
Histoplasmosis: Histoplasma antigen EIA

- serum (92.3% sens.) & urine (100% sens)



Histoplasmosis	Sensitivity (%)
Disseminated	90-95
Acute pulmonary	80
Chronic pulmonary	20

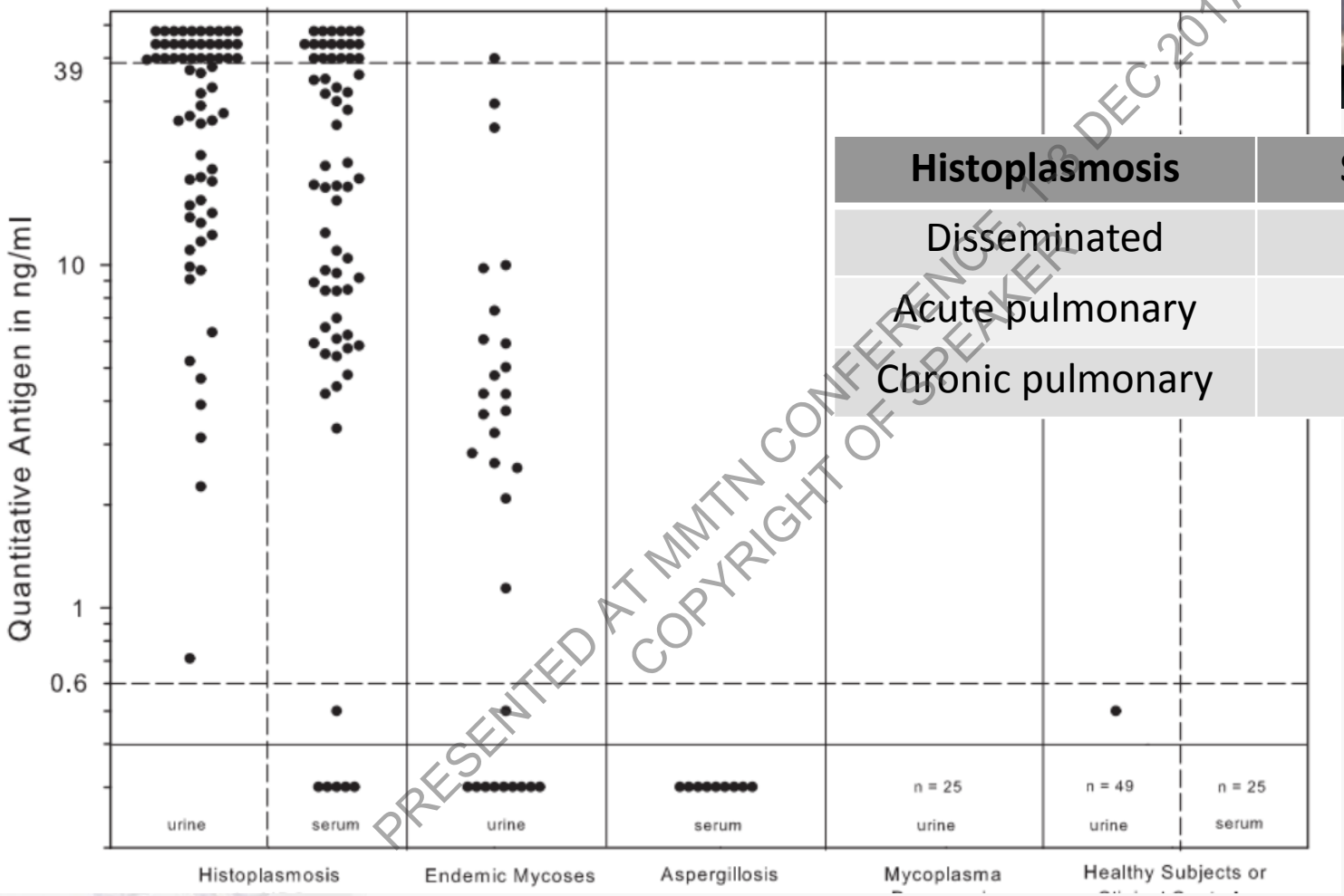


FIG. 2. Sensitivity in disseminated histoplasmosis in samples from patients with AIDS and specificity in samples from controls. The antigen concentration, in nanograms/milliliter, is shown on the vertical axis. The sensitivity in urine was 100%, and in serum it was 92.3%; the specificity was 99.0% in controls without fungal infections.

Connolly *et al*. Clin Vaccine Immunology 2007

Candidiasis: Mannan

The use of mannan antigen and anti-mannan antibodies in the diagnosis of invasive candidiasis: recommendations from the Third European Conference on Infections in Leukemia

Małgorzata Mikulska,[✉1] Thierry Calandra,[2] Maurizio Sanguinetti,[3] Daniel Poulain,[4] and Claudio Viscoli[5], the Third European Conference on Infections in Leukemia Group

Crit Care. 2010;14(6):R222

- Investigated in 14 studies that comprised 453 patients and 767 controls
  - specificity(86%)
  - sensitivity (83%)
    - *C. albicans* (80-100%)
    - *C. tropicalis* & *C. glabrata* (60-75%)
    - *C. parapsilosis* &*C. krusei* (40–50%)

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Aspergillosis: Galactomannan

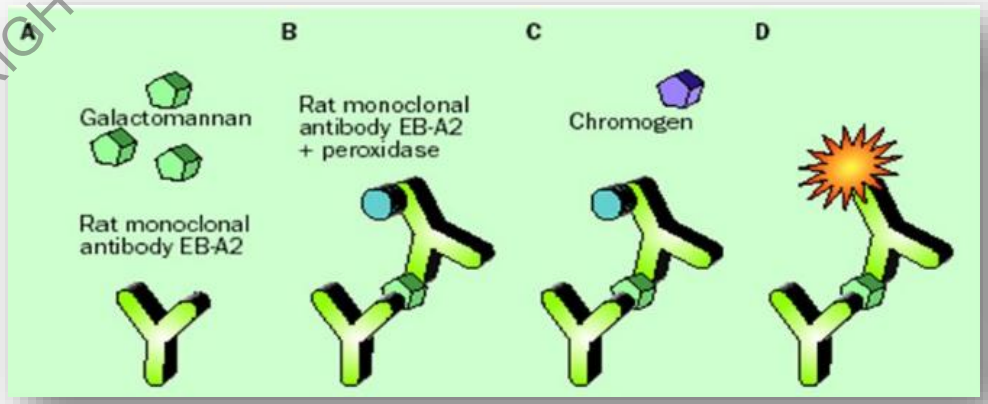
- Major component of fungal cell wall especially *Aspergillus* spp. and also found in
  - Family of Trichocomaceae ie. *Penicillium* spp., *Paecilomyces* spp.
  - *Fusarium* spp.
  - *Histoplasma capsulatum*
  - *Pneumocystis jirovecii*

Tortorano et al. J Clin Microbiol 2012, Huang et al. AIDS 2007, Wheat et al. Clin Vaccine Immunol 2007

- Platelia Aspergillus EIA (Bio-Rad, France)
  - Sandwich ELISA
  - Anti-GM monoclonal antibody : mAb EB-A2

    (Marisa et al. Sem in Res and Critical care Med 2015)
  - FDA approved : as a diagnostic aids for invasive aspergillosis (IA)

    (FDA. May 2003)



www.slideplayer.com

- Clinical samples interpretation

	Recommended cut-off	Collected samples	Sens.	Spec.	Reference
Serum	Index 0.5 Index 1.0	• 2 aliquots: same positive sample + 1 sample collected at a different time point • 1 single sample	60-80%	80-95%	*Marisa 2015* *Lamoth 2016*
BAL	Index 0.5-1.0	• 2 aliquots of a single BAL fluid sample	85-90%	90-95%	*Guo Y L et al. 2010* *Zou M et al. 2012*
CSF*	Index 0.5-2.0	• 1 single sample	85-90%	95-100%	*Chong GM et al. 2016*

- *CSF is validated only for cerebral aspergillosis diagnosis by EORTC-MSG and ECIL so using under expertise consultation is recommended* (De Pauw B et al. 2008, Marchetti O et al. 2012)
- *CSF has not been approved by FDA yet.*

- Two main strategies of use for serum sample:
  - Serial collection of samples (2-3 times/week) in high risk patients
  - Intensive testing in symptomatic patients

Marisa et al. Sem in Res and Critical care Med 2015, Lamoth et al. J of fungi 2016

GM detection in other specimens

Urine *(Duettmann W et al. Med Mycol 2014)*

- Study in 75 proven IA cases underlying hematological malignancies
- GM index cut-off : 0.5
  - 47.6% Sensitivity
  - 86% Specificity
  - 24.4 Positive predictive value (PPV)
  - 94.5 Negative predictive value (NPV)

Moreover, GM positive also reported in

- Cyst fluids in patients with polycystic kidney disease *(Miller-Hjelle MA et al. Emerg Infect Dis 1997)*
- Subphrenic abscess in a pediatric patient with proven IA underlying chronic granulomatous *(Verweij PE et al. J ClinMicrobiol 2000)*
- Purulent material, study in patients with fungal rhinosinusitis *(Klont RR et al. Clin Infect Dis 2004)*

However, the study of GM in other specimen is very limited, using for routine diagnosis is not recommended.

!! Caution !! GM Negative was found in some species of *Aspergillus* !!

Features of selected non-*A. fumigatus* infections *(modified from Lass-Florl et al. J Antimicrob Chemother 2017)*

Species	Diseases	Specific characteristics
Emericella nidulans	IA in CGD	• More virulent than A. fumigatus • Higher mortality • Propensity to spread from the lung to adjacent structures and to disseminate • Intrinsic resistance to amphotericin B
Emericella quadrilineata	IA in CGD and IA	• Resistant to caspofungin?
Aspergillus calidoustus	IA	• Propensity to disseminate • Intrinsic resistance to azoles • Intrinsic resistance to caspofungin?
Aspergillus terreus	IA	• Propensity to disseminate (63%) • Intrinsic resistance to amphotericin B
Aspergillus tubingensis	IA, airway colonization and ear infections	• Acquired resistance to azoles • Lower propensity to disseminate (10%-30%)
Aspergillus lentulus	IA	• Resistant to azoles and echinocandins • Resistant to amphotericin B
Aspergillus alliaceus	**IA**	**• GM negative** **• High MICs of amphotericin B and caspofungin**
Aspergillus carneus	**IA**	**• GM low positive**
Aspergillus novofumigatus	IA	• Resistant to azoles
Aspergillus alabamensis	Mainly airway colonization	• Resistant to amphotericin B
Aspergillus ustus	IA	• Resistant to amphotericin B, azoles and echinocandins
Aspergillus felis	IA	• High MICs against voriconazole and caspofungin

IA, invasive aspergillosis; CGD, chronic granulomatous disease; GM, galactomannan.

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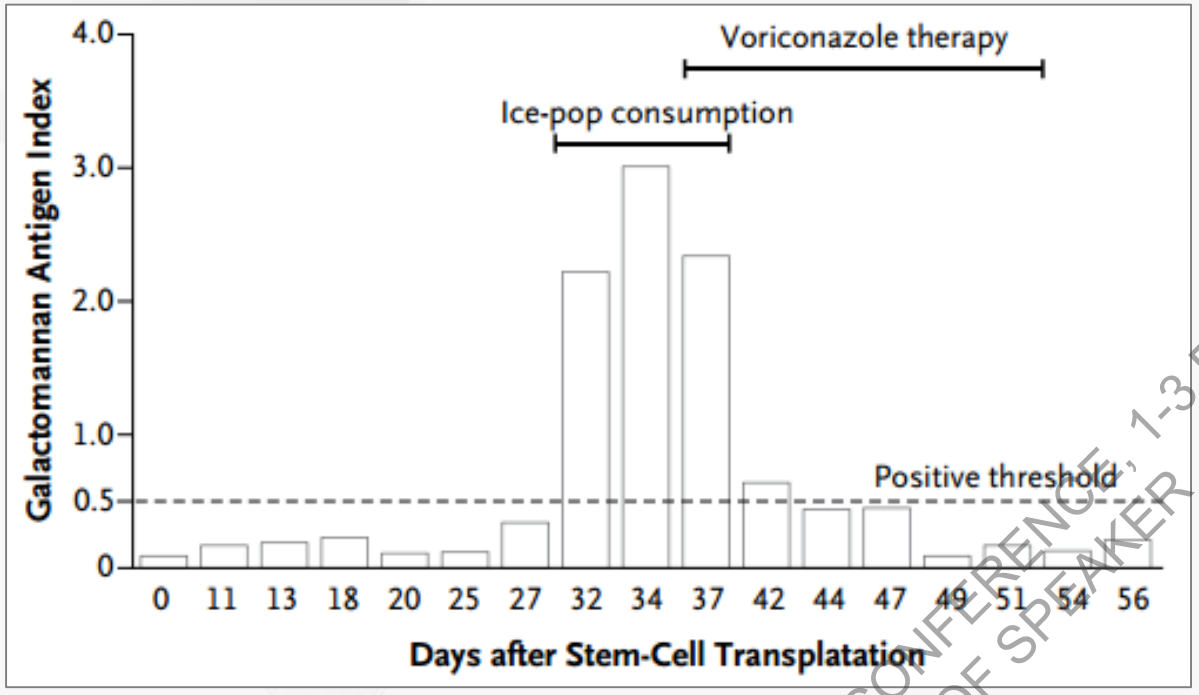
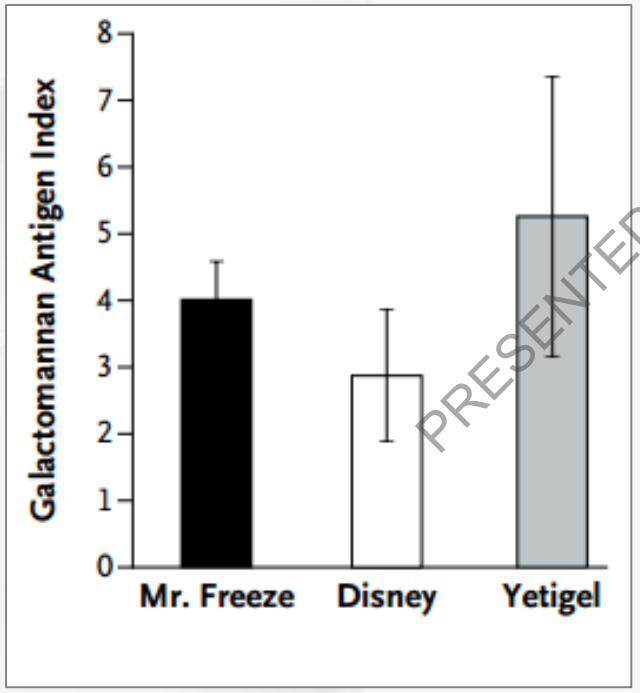
!! Caution !! False positive galactomannan test after ice pop ingestion !!

- A 42-year-old woman
- Received an HLA-matched hematopoietic stem-cell transplant from an unrelated donor for a myeloproliferative syndrome.
- Serum aspergillus antigen (GM) assessed twice weekly from the day of transplantation.
- GM index increased to 2.22 and 3.01 on days 32 and 34, respectively.
- Severe gastrointestinal GVHD.
- Afebrile with no pulmonary or sinus symptoms.
- CT scans of the lung, brain, sinus and abdomen = normal
- Cultures of sputum samples = negative
- PCR blood for aspergillus = negative

Guigue et al. The New England Journal of Medicine. 2013





- 3/37 of ice-pop
  - Positive *Penicilium* spp.
  - showed positive GM
- In ice-pop
  - Sod. gluconate (Thickener & stabilizer for water based frozen dessert in Europe & US)

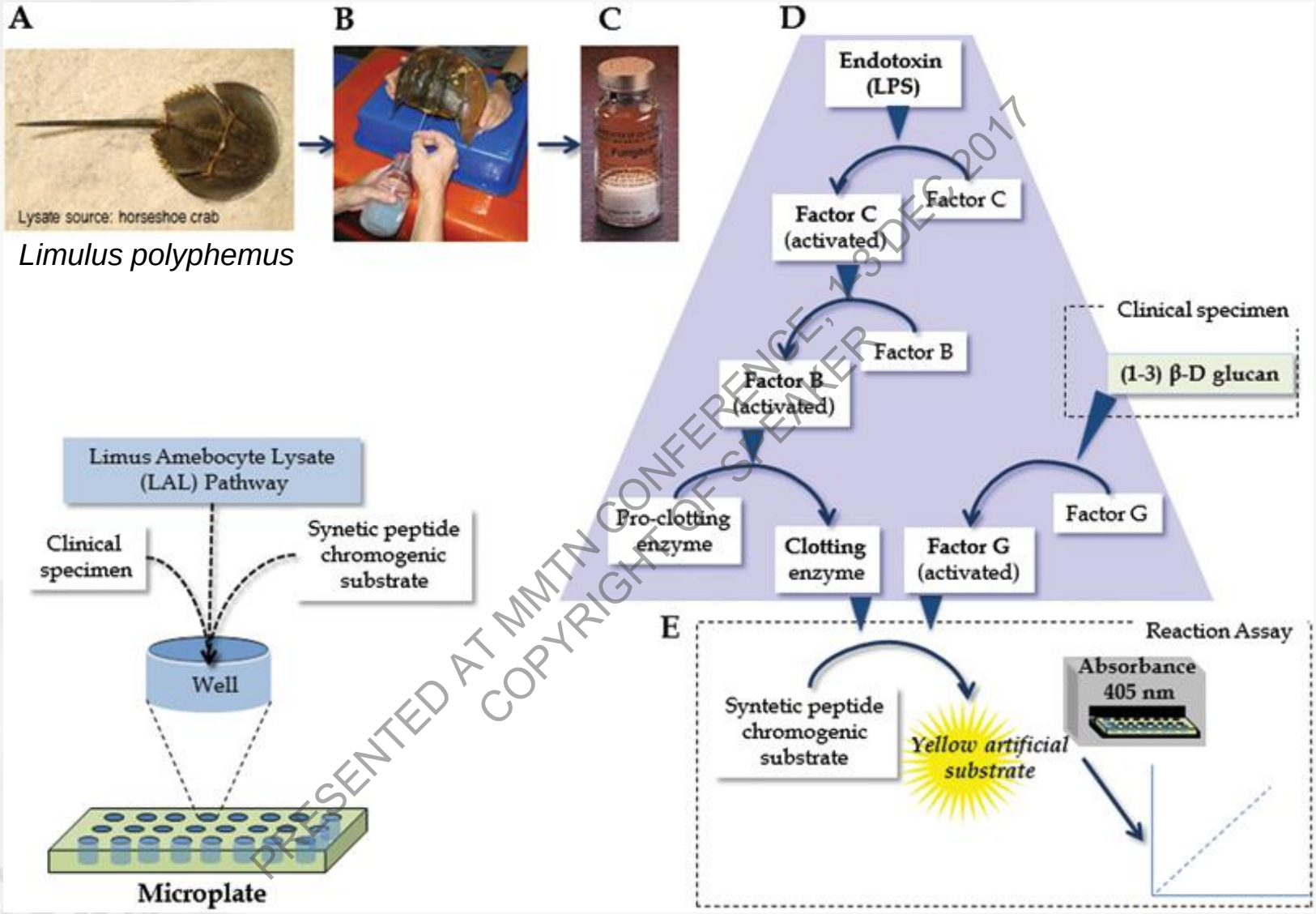
BD: 1,3-β-D-glucan

- Major component of fungal cell wall but less in
  - Mucorales ie. *Mucor* spp., *Rhizopus* spp.
  - *Cryptococcus* spp. and some other Basidiomycota ie. *Malassezia* spp.

Commercial assay	Manufacturer	Horseshoe crab substrate	Detection system	Manufacturer cut-off value	Available
Fungitell assay (Glucatell)	Associated of Cape Cod Inc., East Falmouth, MA, USA	*Limulus polyphemus*	Colorimetric	60-80 pg/ml	US (FDA approved in 2003) and Europe
Fungitec-G test MK (G-MK)	Seikagaku Corporation. Tokyo, Japan)	*Tachypleus tridentatus*	Colorimetric	20 pg/ml	Japan
Beta-glucan test Wako	Wako Pure Chemicals Industries Ltd., Osaka, Japan	*Tachypleus tridentatus*	Turbidimetric	11 pg/ml	Japan
BGSTAR beta glucan test Maruha	Maruha Nichiro Foods Inc.Tokyo, Japan	*Tachypleus tridentatus*	Colorimetric	11 pg/ml	Japan

(Marisa et al. Sem in Res and Critical care Med 2015)

- Fungitell assay (Cape Cod Inc., USA)
  - FDA approved as an aid to diagnose deep-seated mycoses and fungimia.
  - European medical center : presumptive diagnosis of invasive fungal disease
    (Marisa et al. Sem in Res and Critical care Med 2015)
  - The EORTC-MSG panel : included a positive BD test as a microbiological criterion of IFI
    (Lamoth et al. J of fungi 2016)



Biological cascade-based assay

Measuring activation of Factor G through horseshoe crab substrates

- Clinical specimen *(Lamoth et al. J of fungi 2016)*

	Sensitivity	Specificity	Note
Serum	60-80%	80-95%	FDA approved
BAL	85-89%	86-95%	
CSF	85-90%	95-100%	

- Unfortunately, BD is not pathogen specific and cannot differentiate fungal species.
- Major limitations : its low specificity and frequent occurrence of false-positive reaction
  - Non-fungal infection
    - *Pseudomonas* spp.
    - *Streptococcus* spp.
  - Non-infectious disease conditions
    - Hemodialysis with cellulose membranes
    - Albumin transfusion
    - Intravenous immunoglobulin
    - Gauze packing of serosal surfaces
    - Use of cellulose filters for intravenous administration
    - Intravenous amoxicillin-clavulanate

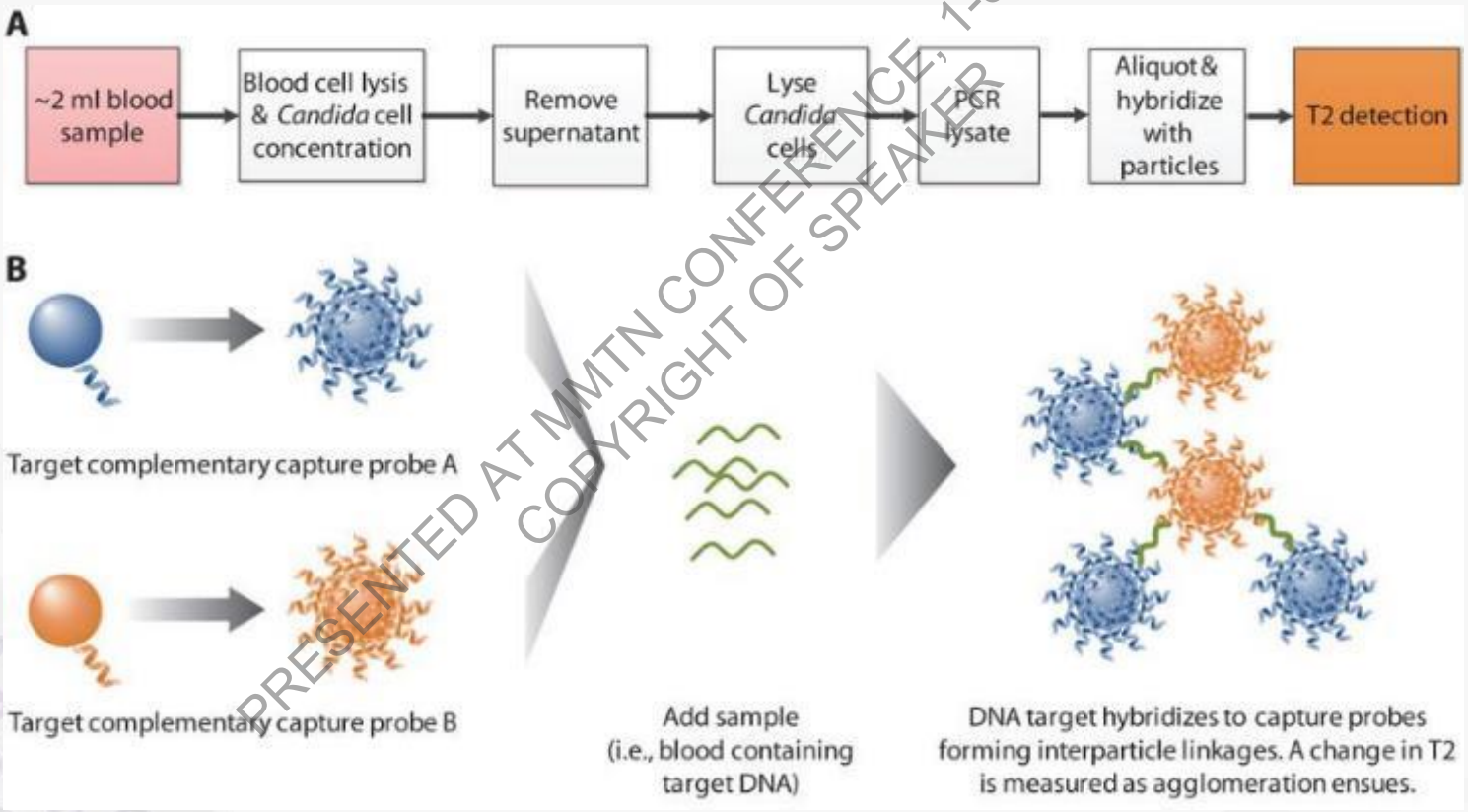
(Marty et al. Med Mycol 2009, Mennink-Kersten MA et al. N Eng J Med 2006, Mennink-Kersten MA et al. Clin Infect Dis 2008)

Sci Transl Med. 2013 Apr 24;5(182):182ra54. doi: 10.1126/scitranslmed.3005377.

T2 magnetic resonance enables nanoparticle-mediated rapid detection of candidemia in whole blood.

Neely LA[1], Audeh M, Phung NA, Min M, Suchocki A, Plourde D, Blanco M, Demas V, Skewis LR, Anagnostou T, Coleman JJ, Wellman P, Mylonakis E, Lowery TJ.

Diagnostic detection method utilizing miniaturized magnetic resonance technology, measures how water molecules react in the presence of magnetic fields.



Moreover, T2MR also can detect the antigen directly from hemoculture.

Antibody Detection

- Usually uses in endemic mycoses: Histoplasmosis, Sporotrichosis, Coccidioidomycosis *etc.* in immunocompetent hosts
  - High Specificity
  - Sensitivity depends on the phase of disease
- But, not useful for opportunistic fungal infections in immunocompromised hosts

Molecular Technology for Fungal Identification & Diagnosis

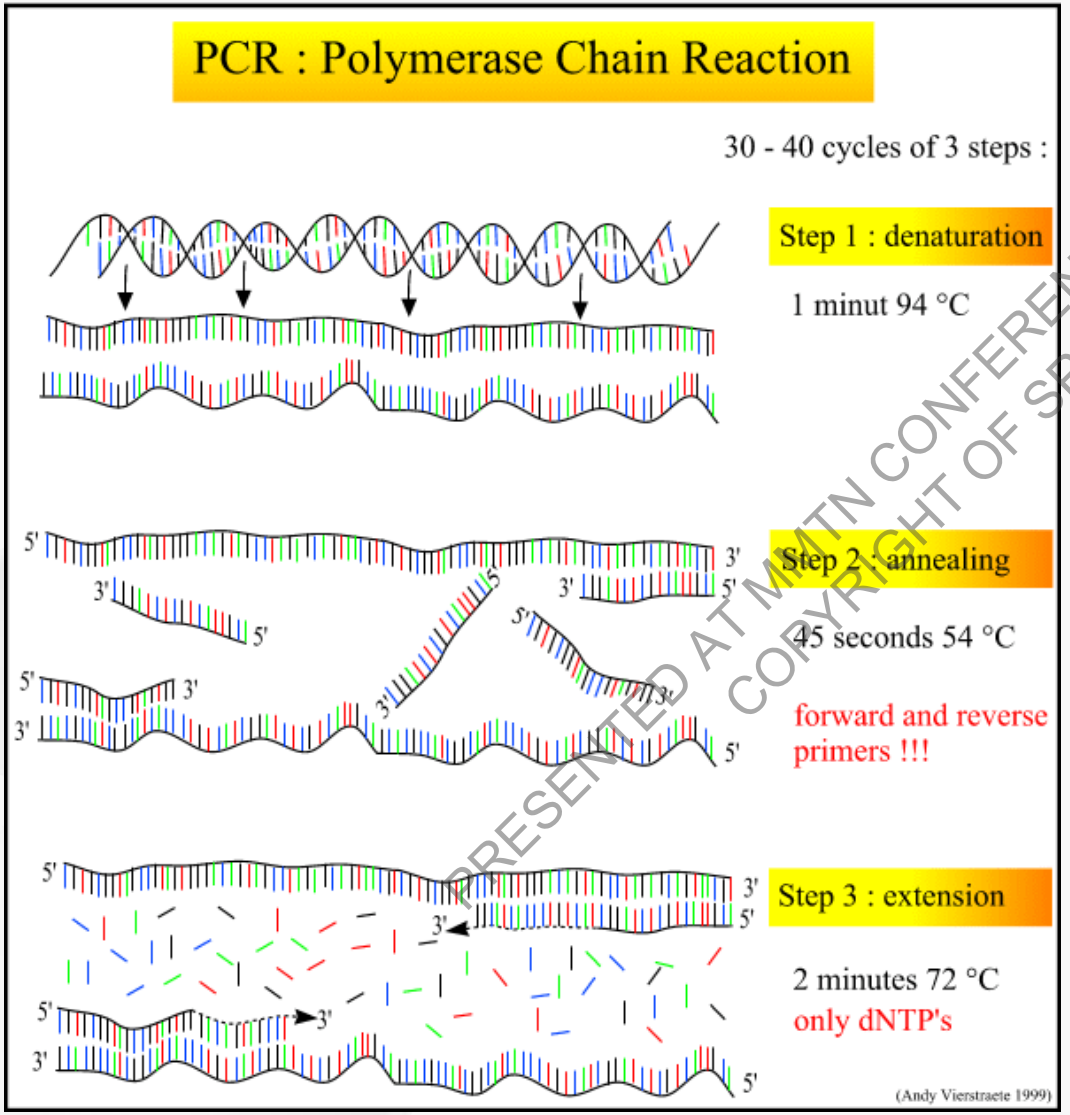
Case 1

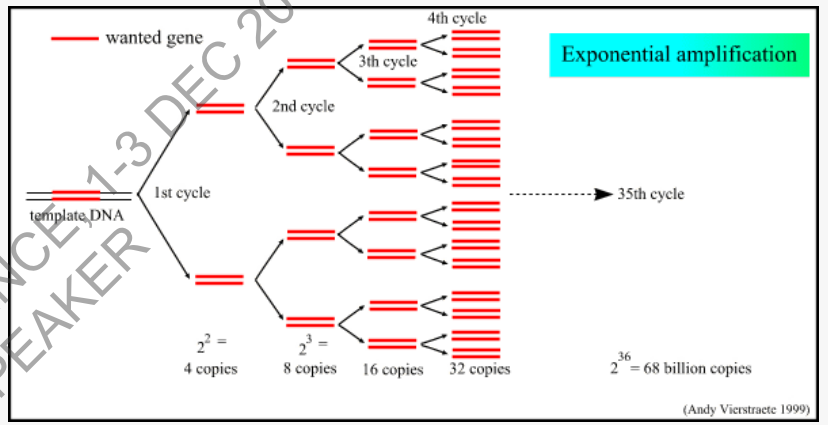
- 43 years-old, female
- Headache, 1 month
- Dx: Brain abscess
- Specimen: Pus
- Request for:
  - KOH prep. >> yeast + hyphae
  - Culture >> no fungal growth

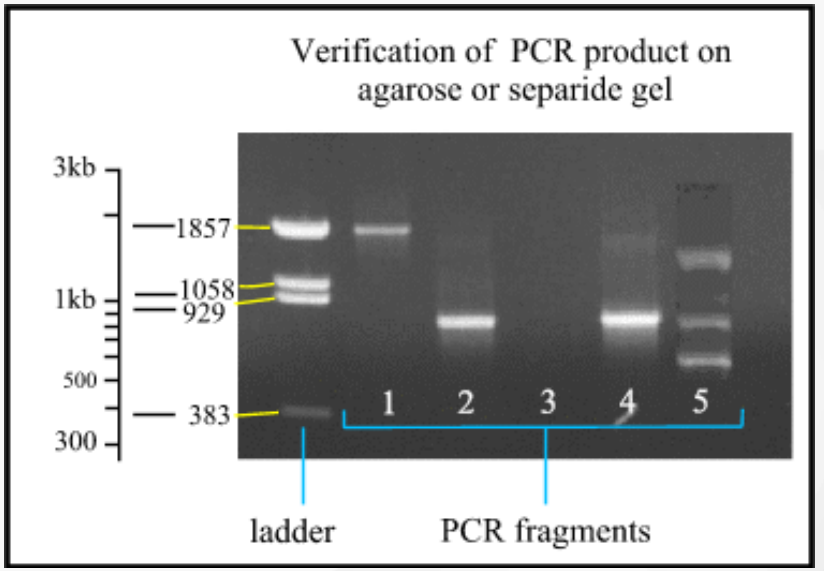
- 47 years-old, male
- Dx: Subcutaneous mycosis
- Specimen: Tissue
- Request for:
  - KOH prep. >> Dark septate hyphae
  - Culture (24d) >> Non-sporulated dark septate hyphae

Molecular Technology

(genetic-based study / PCR: polymerase chain reaction)







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Molecular Technology

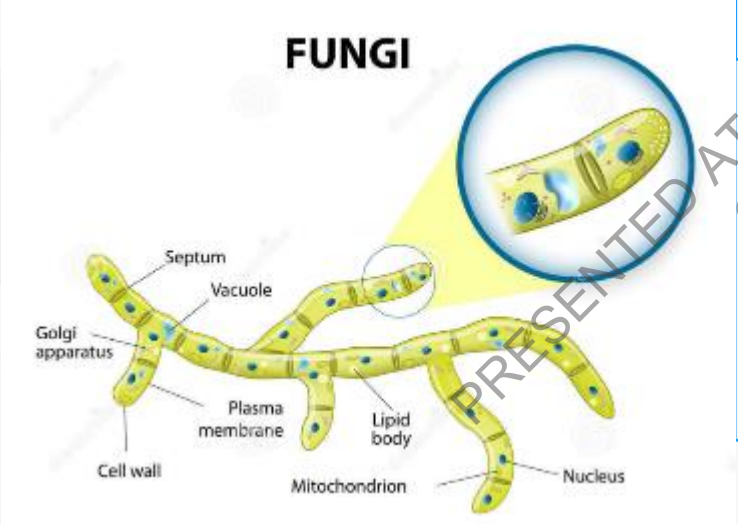
Purpose: genotyping

Evolution & Phylogenetic Epidemiology

Identification & diagnosis

DNA sequence; direct

DNA sequence; indirect SNPs



Nuclear ribosomal DNA (rDNA)

Whole genome of fungi varies from 8.97 Mb to 177.57 Mb

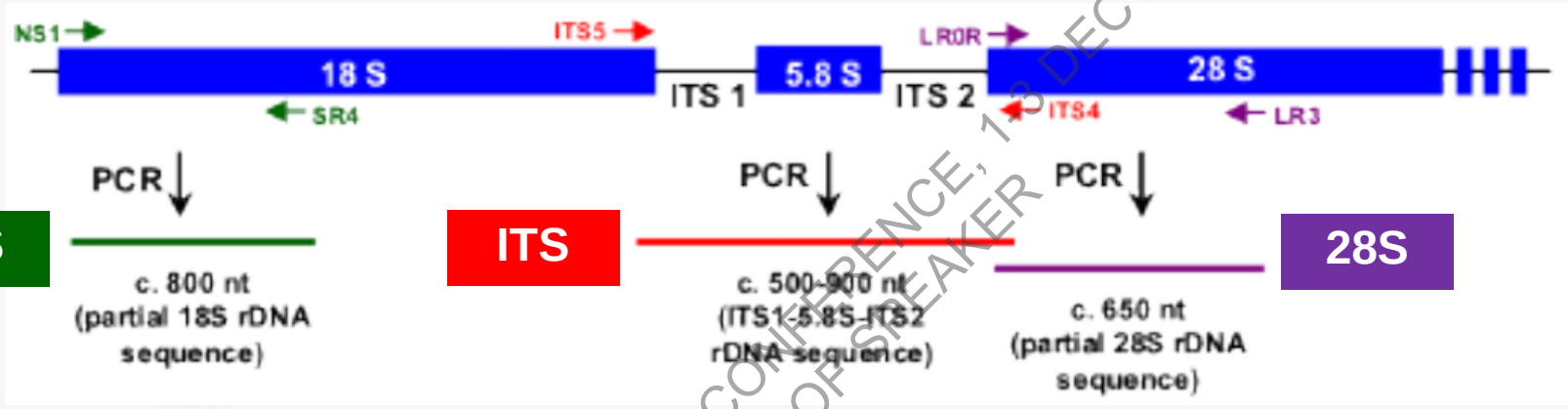
Other gene *ie*.

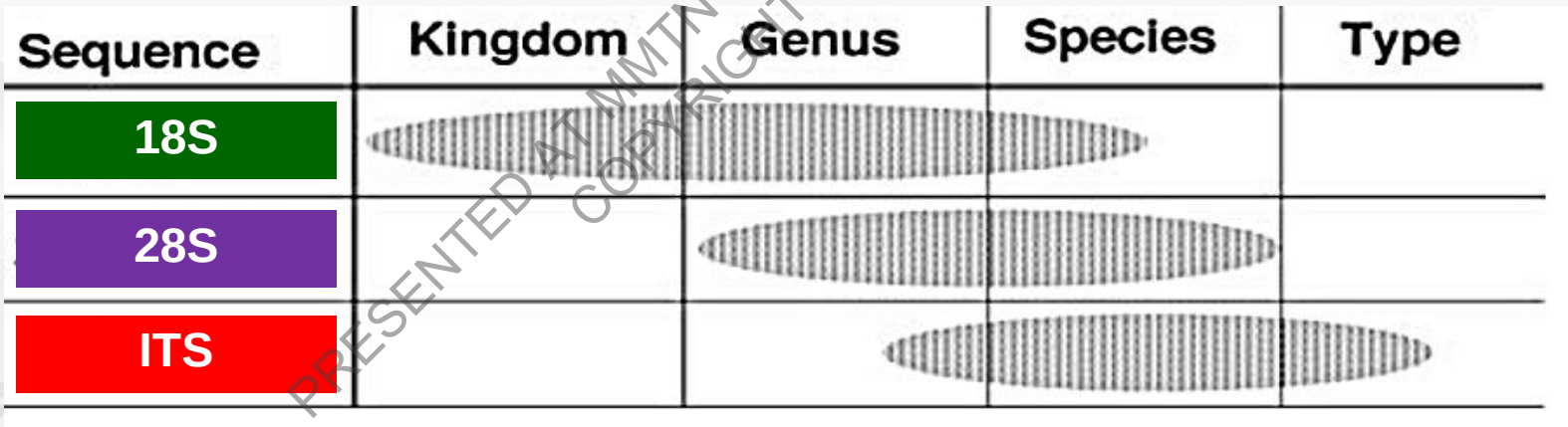
- *Chitin synthase* II
- *Cytochrome oxidase* II
- *Tubulin-beta*

Nuclear ribosomal DNA

Compose of

1. 18S rDNA
2. Large subunit of rDNA (LSU) or 26s rDNA
3. Internal transcribed spacer region (ITS: ITS 1 and ITS2)
4. Intergenic spacer region (IGS)





Localization of universal primer currently used for fungal identification

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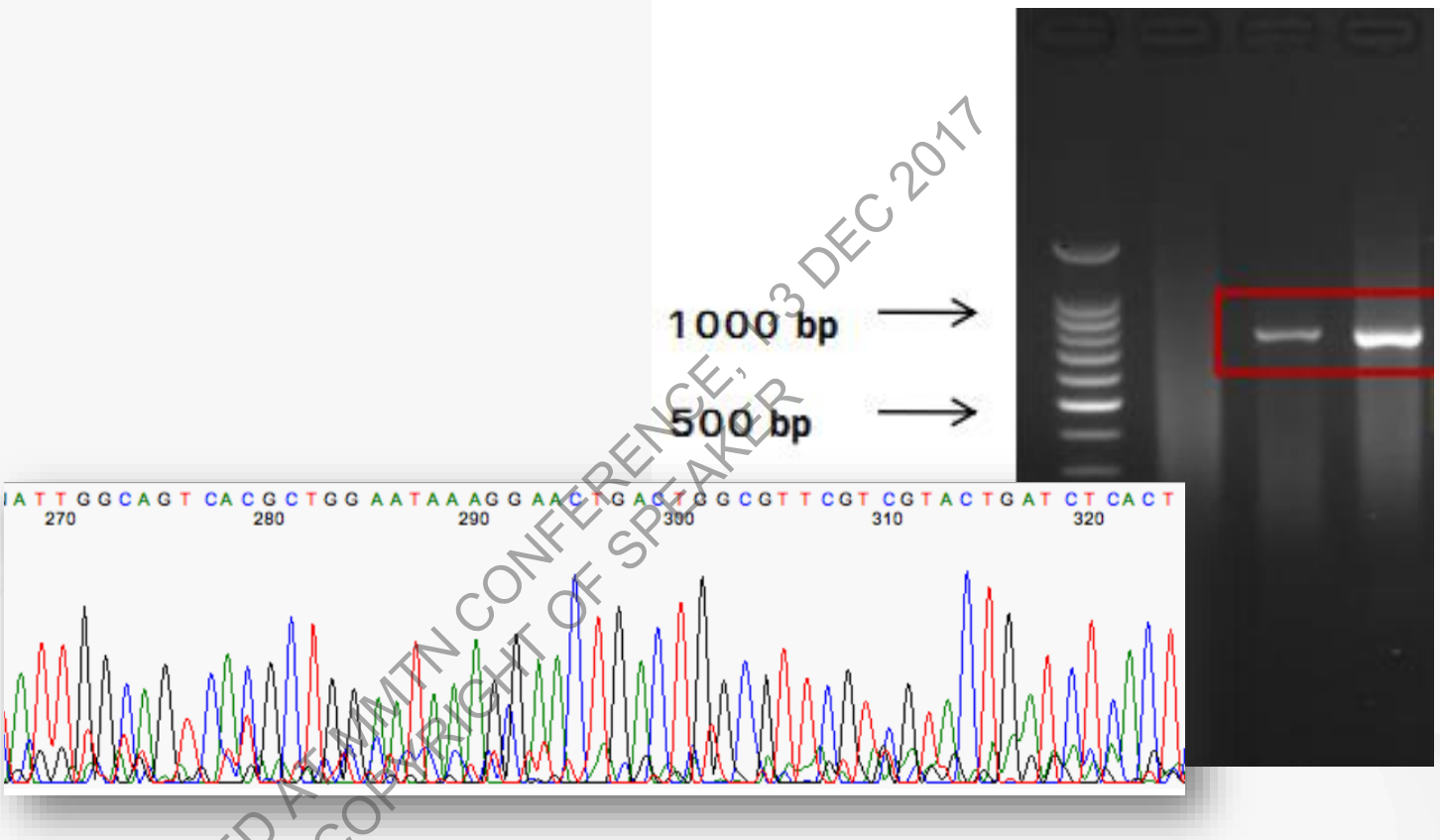
J.B. Stielow et al. ***One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes*** *Persoonia. 2015 Dec; 35: 242–263.*

DNA extraction from PUS

PCR amplification and Sequencing process



Primer LRR/LR3

ggaggaaaag aaaccaacag ggattgcctt agtagcggcg agtgaagcgg caaaagctca
aatttgaaat ctggcacttt cagtgtccga gttgtaattt gaagaaggta tctttgggtc
tggctcttgt ctatgtttct tggaacagaa cgtcacagag ggtgagaatc ccgtgcgatg
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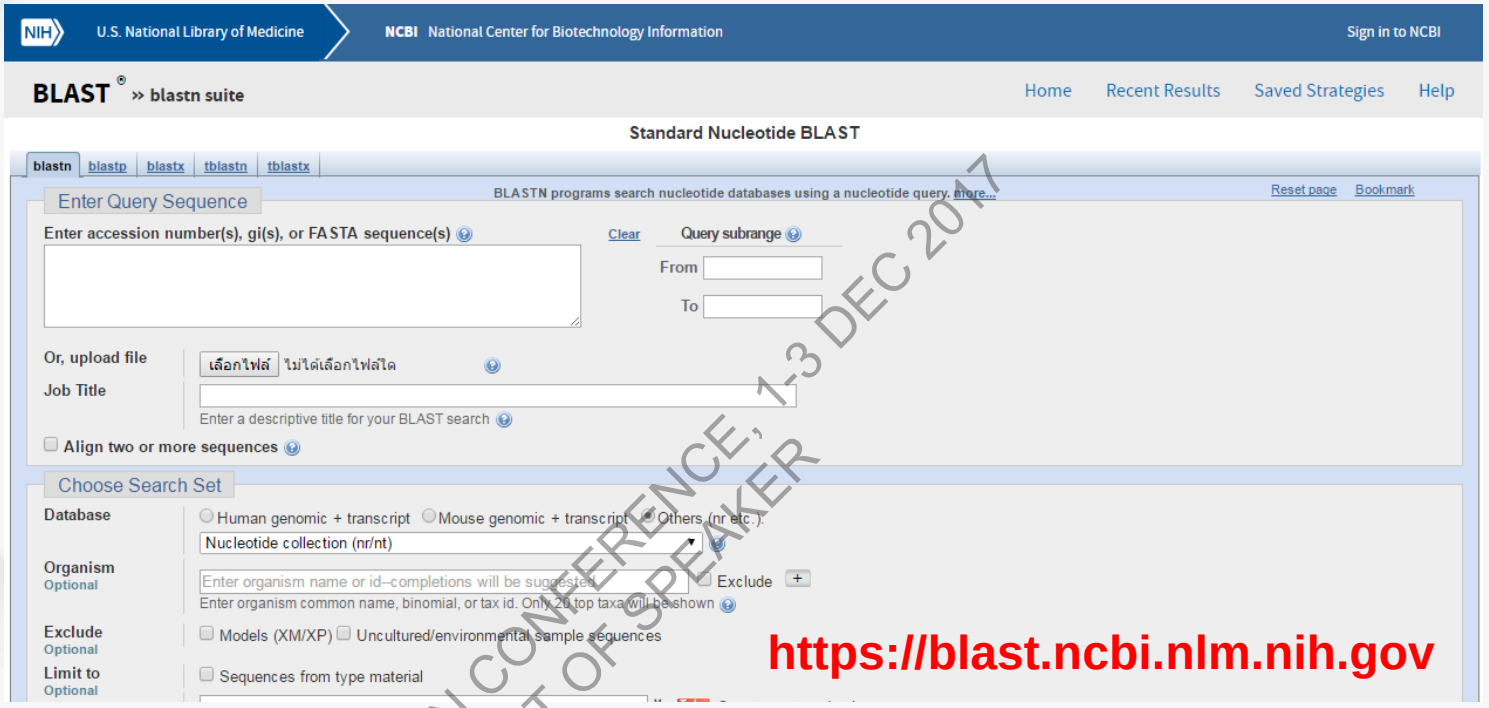
tacagtgatg gaaagatgaa aagaactttg aaaagagagt gaaaaagtac gtgaaattgt
tgaaagggaa gggcttgaga tcagacttgg tattttgtat gttactctct cgggggtggc
ctctacagtt taccgggcca gcatcagttt gagcggtagg ataagtgcaa agaaatgtgg
cactgcttcg gtagtgtgtt atagtctttg tcgatactgc cagcttagac tgaggactgc
ggcttcggcc taggatgttg gcataatgat cttaagtcgc ccgtcttga

DNA extraction from PUS

PCR amplification and Sequencing process

Blast to database



https://blast.ncbi.nlm.nih.gov

Basic Local Alignment Search Tool

Submitted sequence

DataBank

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10/1/2014

ISHAM ITS Database

NCBI/ BLAST/ blastn suite/ Formattin
Formatting options
Download
Blast report description

Nucleotide Sequence (551 letters

RID 2RB0MZCK015
Query ID lcl|31225
Description None
Molecule type nucleic acid
Query Length 551

Distribution of 100 Bla

Color

#	Reference description	Score	Probability	Similarity%	Fragments	Overlap%	Direction
1	MITS984, Candida parapsilosis, N437: ITS sequence ITSSeq	741.762	0	99.787	1	100	+/+
2	MITS985, Candida parapsilosis, N437: ITS sequence ITSSeq	741.762	0	99.787	1	100	+/+
3	MITS941, Candida parapsilosis, N437: ITS sequence ITSSeq	741.762	0	99.787	1	100	+/+
4	MITS951, Candida parapsilosis, N437: ITS sequence ITSSeq	741.762	0	99.787	1	100	+/+
5	MITS986, Candida parapsilosis, N437: ITS sequence ITSSeq	741.762	0	99.787	1	100	+/+
6	MITS989, Candida parapsilosis, N437: ITS sequence ITSSeq	741.762	0	99.787	1	100	+/+

Results	ITS	
PCR	Positive	500 bp
Sequence	*Candida parapsilosis*	99.8% similarity

Description	Max score	Total score	Query cover	E value	Ident	Accession
Candida parapsilosis strain 34094hse 28S ribosomal RNA gene, partial sequence	1018	1018	100%	0.0	100%	KJ817164.1
Candida parapsilosis strain 29936hse 28S ribosomal RNA gene, partial sequence	1018	1018	100%	0.0	100%	KJ817153.1
Candida parapsilosis strain 24853hse 28S ribosomal RNA gene, partial sequence	1018	1018	100%	0.0	100%	KJ817128.1
Candida parapsilosis strain 2500096400sam 28S ribosomal RNA gene, partial sequence	1018	1018	100%	0.0	100%	KJ817094.1
Candida parapsilosis strain 78981hse 28S ribosomal RNA gene, partial sequence	1018	1018	100%	0.0	100%	KJ817088.1

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Fereydounia khargensis: A new and uncommon opportunistic yeast from Malaysia

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Fereydounia khargensis represents a new lineage in the order Urocystales, subphylum Ustilaginomycotina. It was first discovered in Iran in 2014;[1] in 2016, RM Tap et al reported 2 invasive infections caused by *F. khargensis* in immunocompromised patients in Malaysia, which are discussed below.[2]

The first case of *F. khargensis* was from an HIV-positive patient. He was admitted because of episodes of fever associated with chills and rigors for 2 weeks. The patient was initially treated with amphotericin B, but the clinical condition did not improve. Antifungal treatment was changed to itraconazole and he was discharged upon improvement.

The second case was a patient was a hepatitis B carrier with hypertension, diabetes mellitus and end-stage renal failure on continuous ambulatory peritoneal dialysis (CAPD). He was admitted because of a dislodged distal connector of his Tenckhoff catheter caused by a fall in the toilet. After the incident, the dislodged connector was reconnected to the Tenckhoff catheter and used for CAPD. Fluconazole was started following yeast growth from the peritoneal fluid. The clinical condition of the patient improved after fluconazole treatment.

Both cases grew yeast-like colonies. Figures 1 and 2 show the macro- and microscopic examinations. Results from API 20C AUX and VITEK 2 identification system were *Cryptococcus neoformans* (98% probability) and *Cryptococcus laurentii* (89% probability), respectively. Internal transcribed spacer (ITS) and D1/D2 region in large subunit (LSU) of rRNA gene were amplified using universal primers and sequenced. Both *F. khargensis* isolates matched 99.7% (ITS) and 100.0 % (LSU) to the reference strain, IBRC-M 30116.

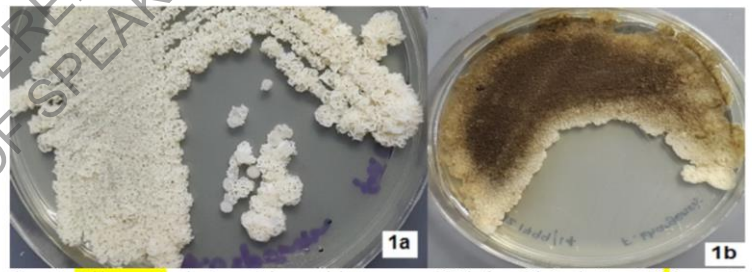

Figure 1. *F. khargensis* colonies on Sabouraud dextrose agar (SDA) after 48 h incubation at 30°C presented as cream-colored colonies, dry and wrinkled (1a). However, after 72 h of incubation, the colonies started producing melanin-like pigmentation, which turned even darker after 120 h (1b).

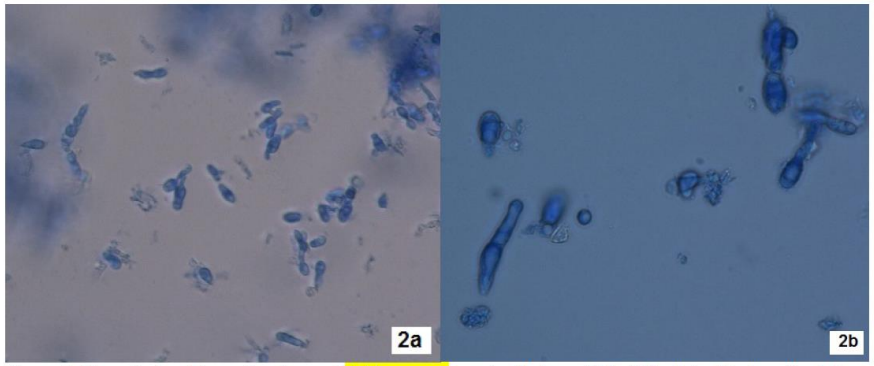

Figure 2. Elongated and irregular shape of *F. khargensis* yeast cells from 48 hr SDA plate. The length of the cells ranged from 5.63 to 18.34 µm. Magnification at x40 (2a) and x100 (2b).

Thank you

Guarner et al. Clin Microbiol Rev. 2011